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Endogenous Substrates for Energy Metabolism in Spermatozoa of the Sea Urchins *Arbacia lixula* and *Paracentrotus lividus*

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Abstract. Energy metabolism was examined in the spermatozoa of the sea urchins *Arbacia lixula* and *Paracentrotus lividus,* which belong to the orders Arbacioida and Echinoida respectively. *P. lividus* spermatozoa contained various phospholipids and cholesterol, and their endogenous triglyceride (TG) content was quite low. After dilution of dry sperm in artificial seawater, the level of phosphatidylcholine (PC) decreased rapidly, but other phospholipids remained at constant levels. In contrast to those of *P. lividus,* the spermatozoa of *A. lixula* contained TG as well as phospholipids and cholesterol. Following incubation of *A. lixula* spermatozoa in artificial seawater, TG decreased, but there were no concomitant changes in the levels of phospholipids. Trace amounts of glycogen were present in both species. High lipase activity was demonstrated in *A. lixula* spermatozoa, but in *P. lividus* spermatozoa lipase activity was low and phospholipase A_2 activity was high. It is thus concluded that *A. lixula* spermatozoa obtain energy for swimming through oxidation of endogenous TG, whereas *P. lividus* spermatozoa use PC as a substrate for energy metabolism. This suggests that the system of energy metabolism in spermatozoa is different in the orders Arbacioida and Echinoida.

Introduction

Sea urchin spermatozoa start their flagellar movement immediately after being spawned into seawater. The flagellar movement results partly from reactions catalyzed by dynein ATPase (Gibbons and Gibbons, 1972; Christen *et al.,* 1982, 1983; Evans and Gibbons, 1986). Thus, energy metabolism to produce ATP is indispensable for swimming. Sea urchin spermatozoa could not use an exogenous substrate for energy metabolism because they swim in seawater, which contains hardly any nutrients. Previous studies in *Echinus esculentus* (Rothschild and Cleland, 1952) and *Hemicentrotus pulcherrimus* (Mohri, 1957a; Mita and Yasumasu, 1983) have shown that the endogenous phospholipid content of spermatozoa decreases following the initiation of flagellar movement. It has also been reported that *H. pulcherrimus* spermatozoa possess fatty acid oxidizing activity (Mohri, 1957b; Mita and Ueta, 1988, 1990). *H. pulcherrimus* spermatozoa generally contain various phospholipids and cholesterol (Ch), and their endogenous triglyceride (TG) and glycogen contents are extremely low (Mita and Yasumasu, 1983; Mita and Ueta, 1988, 1989). The decrease in the phospholipid content of *H. pulcherrimus* spermatozoa during swimming has recently been reported to be caused solely by a change in the level of phosphatidylcholine (PC) (Mita and Ueta, 1988). Similar findings for other sea urchins of the order Echinoida (Mita and Nakamura, 1993) indicate that PC is the main substrate for energy metabolism in these spermatozoa. The hydrolysis of PC in preference to other available phospholipids is related to the properties of phospholipase A_2 (Mita and Ueta, 1988, 1990; Mita and Nakamura, 1993).

In *Arbacia lixula* and *Paracentrotus lividus,* which belong to the orders Arbacioida and Echinoida respectively,

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the phospholipid content in the spermatozoa decreases following incubation in seawater (Mohri, 1964). On the other hand, the spermatozoa of *Glyptocidaris crenularis*, a member of the suborder Phymosomatoida of the order Arbacioida, contain TG as well as phospholipids and Ch, and use this endogenous TG to produce energy for swimming (Mita, 1991). This suggests that the spermatozoa of sea urchins in the order Arbacioida have an energy metabolic system different from that in the order Echinoida. Thus, it is still unclear whether the endogenous substrate used for energy metabolism by *A. lixula* spermatozoa is PC, TG, or a combination of the two. To clarify energy metabolism in sea urchin spermatozoa, the present study compared the energy production systems in spermatozoa of *A. lixula* and *P. lividus*.

Materials and Methods

Materials

Sea urchins, *A. lixula* and *P. lividus*, were collected in the Gulf of Napoli, Italy, and induced to spawn by intracoelomic injection of 0.5 *M* KCl. Semen was always freshly collected as "dry sperm" and kept undiluted on ice. The number of spermatozoa was calculated from the protein concentration, which was determined using a Micro BCA protein assay kit (Pierce, IL). The average protein content per 10^9 spermatozoa was 0.5 ± 0.1 mg in both species.

Incubation of spermatozoa

Dry sperm were diluted 100-fold in artificial seawater (ASW) consisting of 458 m*M* NaCl, 9.6 m*M* KCl, 10 m*M* $CaCl_2$, 49 m*M* $MgSO_4$, and 10 m*M* Tris-HCl at pH 8.2. After dilution and incubation for 1 h at 20°C, the sperm suspension was centrifuged at 3000 × *g* for 5 min at 0°C.

In a cell-free system, dry sperm were homogenized with 10 m*M* $CaCl_2$, 10 m*M* $MgCl_2$, 1 m*M* dithiothreitol, and 50 m*M* Tris-HCl at pH 7.5. The homogenate was incubated for 1 h at 20°C.

Assay of glycogen and glucose content

Before and after incubation of dry sperm in ASW, samples of spermatozoa were homogenized with 0.6 *M* perchloric acid. The glycogen content of the homogenate was determined by an enzymatic method (Keppler and Decker, 1984). The acidified homogenate was centrifuged at 10,000 × *g* for 10 min at 4°C, and the supernatant was used for the estimation of glucose after neutralization to pH 6.5–7.0 with KOH. Glucose was measured enzymatically according to the method of Kunst *et al.* (1984).

Analysis of lipids

The total lipid content of the spermatozoa was extracted by the method of Bligh and Dyer (1959) and analyzed by high-performance thin-layer chromatography (HPTLC), according to the method of Macala *et al.* (1983) with some modification, as previously described (Mita and Ueta, 1988, 1989). The amounts of PC, phosphatidylserine (PS), phosphatidylethanolamine (PE), cardiolipin (DPG), TG, Ch, and free fatty acid (FFA) in the sea urchin spermatozoa were determined densitometrically from the standard curves of the respective authentic lipids.

Estimation of lipase and phospholipase activity

Dry sperm were washed twice with 0.21 *M* mannitol, 0.07 *M* sucrose, and 10 m*M* Tris-HCl at pH 7.5 before being homogenized with 10 m*M* $CaCl_2$, 10 m*M* $MgCl_2$, 1 m*M* dithiothreitol, and 50 m*M* Tris-HCl at pH 7.5. The homogenate was incubated with 4.6 kBq [carboxyl-^{14}C]triolein (4.1 GBq/mmol), 2.3 kBq 1-palmitoyl-2-[1-^{14}C]arachidonyl-PC (1.9 GBq/mmol) or 2.3 kBq 1-palmitoyl-2-[1-^{14}C]arachidonyl-PE (1.9 GBq/mmol) for 1 h at 20°C in a total volume of 0.4 ml. The lipids were extracted according to Bligh and Dyer (1959). The radioactivity in the FFA fraction was separated by thin-layer chromatography (TLC) and measured by liquid scintillation spectrometry.

Oxygen consumption

Oxygen consumption in a sperm suspension was measured polarographically with an oxygen consumption recorder (Gilson 5/6H oxygraph, WI). Eighteen microliters of dry sperm (1–2 × 10^9 sperm) was incubated in 1.8 ml ASW in the closed vessel of the oximeter at 20°C. The concentration of saturated oxygen in ASW at 20°C was 234 nmol O_2/ml (Mita and Yasumasu, 1983). The diluted spermatozoa were left exposed to air until their oxygen consumption was determined. Total oxygen consumption was calculated from the respiratory rate and incubation period, as described previously (Mita and Yasumasu, 1983).

Reagents

The lipid standards were purchased from Sigma Chemical (St. Louis, MO). [Carboxyl-^{14}C]triolein, 1-palmitoyl-2-[1-^{14}C]arachidonyl-PC and 1-palmitoyl-2-[1-^{14}C]arachidonyl-PE were obtained from Du Pont–New England Nuclear (Wilmington, DE). All reagents and solvents were of analytical grade. HPTLC and TLC plates (silica gel 60) were obtained from E. Merck (Germany).

Statistical analysis

All data are expressed as means ± SEM for several experiments. Data were statistically analyzed by the Student's *t* test.

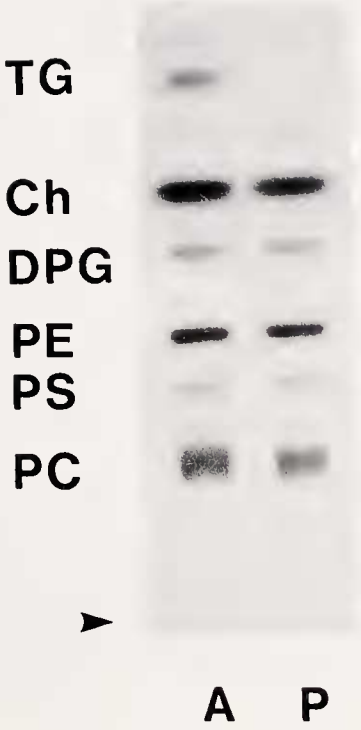

Figure 1. High-performance thin-layer chromatogram of sea urchin sperm lipids. Total lipids were extracted from dry sperm of *Arbacia lixula* (A) and *Paracentrotus lividus* (P) and 10 μg of each were applied to the HPTLC plate. Arrow shows origin.

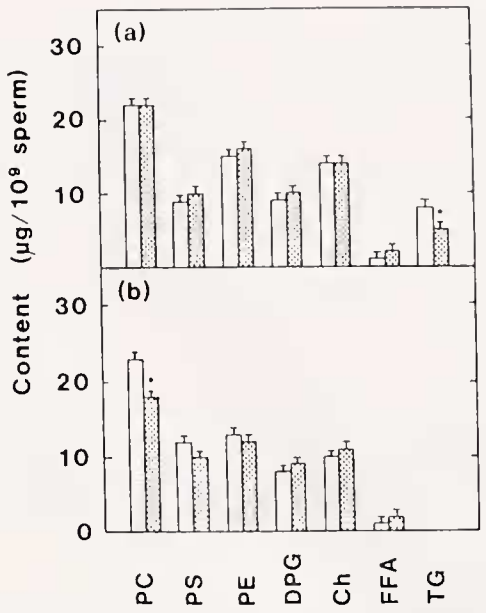

Figure 2. Changes in lipid levels after incubation of *Arbacia lixula* (a) and *Paracentrotus lividus* (b) spermatozoa. Before (clear) and after (dotted) dilution and incubation of dry sperm in seawater for 1 h at 20°C, lipids were extracted and analyzed by HPTLC. Each value is the mean of four separate experiments. Vertical bars show SEM. Data were analyzed statistically by Student's *t* test. **P* values are comparisons with dry sperm values ($P < 0.1$).

Results

Changes in lipid levels after incubation with seawater

The lipids in the spermatozoa of *P. lividus* comprised several kinds of phospholipid and Ch (Fig. 1). Among phospholipids, PC, PS, PE, and DPG were identified in the spermatozoa. Similar phospholipids and Ch were detected in *A. lixula* spermatozoa (Fig. 1). TG was also present at high concentrations in the *A. lixula* spermatozoa, whereas only a trace amount (<1 μg/10^9 sperm) was detectable in those of *P. lividus*.

When the dry sperm of *A. lixula* were diluted and incubated with ASW for 1 h at 20°C, the TG content decreased from 8 ± 1 μg/10^9 sperm to 5 ± 1 μg/10^9 sperm following the initiation of flagellar movement (Fig. 2a). The levels of phospholipids and Ch did not change significantly. In contrast, following incubation of *P. lividus* spermatozoa in ASW, PC decreased from 23 ± 1 μg/10^9 sperm to 18 ± 1 μg/10^9 sperm, with no concomitant change in the levels of other phospholipids (Fig. 2b).

Glycogen was present in the spermatozoa of both species, but at extremely low levels (Table I), and only a trace amount of glucose was present (Table I). There were no statistically significant changes during incubation.

Lipid metabolism in a cell-free system

When the homogenate obtained from the dry sperm of *A. lixula* was incubated for 1 h at 20°C, the TG content decreased and the FFA content increased (Table II). Essentially the same situation was found when intact spermatozoa were used (Fig. 2a). However, the levels of PC, PS, PE, DPG, and Ch showed no statistically significant change. On incubating the homogenate from the dry sperm of *P. lividus* for 1 h at 20°C, the PC content decreased (Table III), as it did in the intact spermatozoa (Fig. 2b). A slight decrease in PE content ($P < 0.05$) was

Table I

Change in the level of glycogen and glucose in Arbacia lixula *and* Paracentrotus lividus *spermatozoa*

	Glycogen (μg/10^9 sperm)		Glucose (nmol/10^9 sperm)	
Species	Dry	Incubation for 1 h	Dry	Incubation for 1 h
A. lixula	0.5 ± 0.1	0.4 ± 0.1	tr.	tr.
P. lividus	0.9 ± 0.1	0.8 ± 0.1	tr.	tr.

Dry sperm were diluted 100-fold in ASW and incubated for 1 h at 20°C. Each value is the mean ± SEM obtained from four separate experiments. tr., trace amount (less than 0.1 nmol/10^9 sperm).

Table II

Lipid metabolism in a cell-free system of Arbacia lixula *spermatozoa*

Lipids	Content (μg/mg protein)	
	Control	Incubation for 1 h
PC	39 ± 2	39 ± 2
PS	20 ± 1	21 ± 1
PE	28 ± 1	29 ± 2
DPG	16 ± 1	16 ± 1
Ch	24 ± 1	24 ± 1
FFA	3 ± 1	7 ± 1[b]
TG	11 ± 1	7 ± 1[a]

The homogenate of dry sperm with 10 m*M* $MgCl_2$, 10 m*M* $MgCl_2$, 1 m*M* dithiothreitol, and 50 m*M* Tris-HCl at pH 7.5 was incubated for 1 h at 20°C. Each value is the mean ± SEM obtained from three separate experiments. *P* values are comparisons with the control. [a]$P < 0.05$; [b]$P < 0.01$.

also observed during incubation. The FFA and lysophosphatidylcholine (Lyso PC) contents increased remarkably ($P < 0.001$). However, PS, DPG, and Ch levels did not change significantly. A trace amount of TG was present in *P. lividus* spermatozoa.

Phospholipase and lipase activities

When the homogenate of dry sperm from *A. lixula* was incubated with [carboxyl-^{14}C]triolein for 1 h at 20°C, the radioactivity was transferred from TG to FFA (Table IV). Lipase activity was found in *A. lixula* spermatozoa; however, incubation with 1-palmitoyl-2-[1-^{14}C]arachidonyl-PC or 1-palmitoyl-2-[1-^{14}C]arachidonyl-PE showed that

Table III

Lipid metabolism in a cell-free system of Paracentrotus lividus *spermatozoa*

Lipids	Content (μg/mg protein)	
	Control	Incubation for 1 h
PC	43 ± 2	31 ± 2[a]
Lyso PC	tr.	6 ± 1[b]
PS	23 ± 2	21 ± 1
PE	30 ± 1	24 ± 2[a]
DPG	18 ± 1	20 ± 1
Ch	24 ± 1	23 ± 1
FFA	4 ± 1	25 ± 1[b]
TG	tr.	tr.

The homogenate of dry sperm with 10 m*M* $CaCl_2$, 10 m*M* $MgCl_2$, 1 m*M* dithiothreitol and 50 m*M* Tris-HCl at pH 7.5 was incubated for 1 h at 20°C. Each value is the mean ± SEM obtained from three separate experiments. tr., trace amount (less than 1 μg/10^5 sperm). *P* values are comparisons with the control. [a]$P < 0.05$; [b]$P < 0.001$.

Table IV

Activities of lipase and phospholipase A_2 in Arbacia lixula *and* Paracentrotus lividus *spermatozoa*

Substrate	Activity (nmol FFA released/h/mg protein)	
	A. lixula	*P. lividus*
PC	0.1 ± 0.1	7.9 ± 0.8
PE	tr.	2.4 ± 0.2
TG	1.4 ± 0.3	tr.

Each value is the mean ± SEM obtained from three separate experiments. tr., trace amount (less than 0.1 nmol FFA released/h/mg protein).

only a little radioactivity was transferred to FFA by the action of phospholipase A_2. In the case of *P. lividus* spermatozoa, phospholipase A_2 activity was high, although lipase activity was extremely low (Table IV). The hydrolysis of PE was about one third that of PC. Phospholipase A_2 in *P. lividus* spermatozoa thus appears to have greater substrate specificity for PC.

Oxygen consumption

Because oxygen is required for oxidation of the lipid, the amount of O_2 consumed by the spermatozoa of *A. lixula* and *P. lividus* was measured at various intervals after dilution in ASW (Fig. 3). The rate of O_2 consumption by the spermatozoa of both species decreased gradually during long-term incubation. In a 1-h incubation, 0.24 ± 0.03 μmol O_2 was consumed by 10^9 *A. lixula* spermatozoa

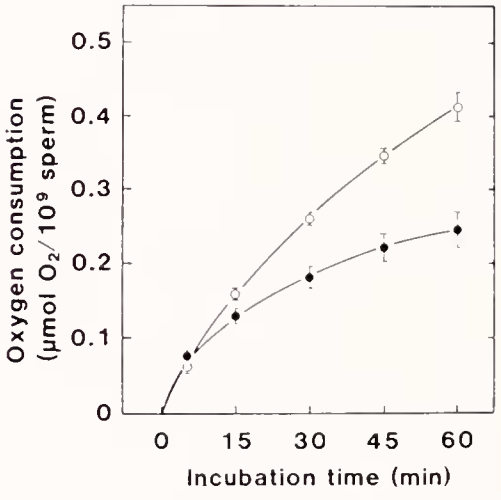

Figure 3. Oxygen consumption in *Arbacia lixula* (●) and *Paracentrotus lividus* (○) spermatozoa. Dry sperm were diluted 100-fold and incubated in seawater at 20°C. Each value is the mean of three separate experiments. Vertical bars show SEM.

and 0.41 ± 0.02 μmol O_2 was consumed by the same number of *P. lividus* spermatozoa.

Discussion

This study showed that the spermatozoa of *A. lixula*, which is a sea urchin of the order Arbacioida (suborder Arbacina), contained a high concentration of TG (Fig. 1). After incubation in ASW for 1 h at 20°C, the TG content of *A. lixula* spermatozoa decreased significantly, with no concomitant change in the levels of phospholipids (Fig. 2a). However, these findings are inconsistent with observations from a previous study (Mohri, 1964), which found that the phospholipid content of *A. lixula* spermatozoa decreased after incubation for 8 h at 20°C. The reason for this discrepancy is unclear, but it is possible that the decrease Mohri observed in phospholipid levels was due to contamination with dead spermatozoa during long-term incubation. Our data also showed lipase activity in *A. lixula* spermatozoa (Table IV). In the cell-free system, a considerable amount of TG was consumed during incubation at 20°C (Table II), indicating that TG is hydrolyzed by lipase. This accords with the results obtained from *G. crenularis* spermatozoa of the order Arbacioida (Mita, 1991). We also found trace amounts of glycogen and glucose in *A. lixula* spermatozoa (Table I). Thus, *A. lixula* spermatozoa may obtain energy through oxidation of fatty acids derived from endogenous TG.

In contrast, the spermatozoa of *P. lividus*, which belongs to the order Echinoida, contained only a trace amount of TG (Fig. 1) and showed low lipase activity (Table IV). The TG level is also low (<1 μg/10^9 sperm) in the spermatozoa of other sea urchins of the order Echinoida (Mita and Ueta, 1988, 1989; Mita and Nakamura, 1993). Thus, it seems unlikely that these spermatozoa use TG as a substrate for energy metabolism. After incubation of *P. lividus* spermatozoa in seawater, PC was shown to decrease significantly, whereas there were no significant changes in the levels of other phospholipids (Fig. 2b). These findings confirm previous reports that the spermatozoa of sea urchins of the order Echinoida use PC as a source of energy (Mita and Ueta, 1988; Mita and Nakamura, 1993). Although we found high concentrations of PC in *A. lixula* spermatozoa (Fig. 2a), there was no statistically significant change in the level of PC during incubation. It appears that this failure to utilize PC is due to the low activity of phospholipase A_2 in *A. lixula* spermatozoa (Table IV).

Our results showed that about 5 μg PC was consumed by 10^9 spermatozoa of *P. lividus* during incubation for 1 h at 20°C (Fig. 2b). Most of the fatty acid moieties in the PC of sea urchin spermatozoa consist of 20 carbons (Mita and Ueta, 1988, 1989; Mita and Nakamura, 1993). On this basis, we calculate that about 15 nmol of fatty acid per 10^9 spermatozoa is released from PC during incubation. The oxidation of 15 nmol fatty acid requires about 0.45 μmol O_2 per 10^9 spermatozoa, and this is consistent with the actual amount of O_2 (about 0.41 μmol) consumed during the 1-h incubation of 10^9 *P. lividus* spermatozoa at 20°C (Fig. 3). Similarly, about 4 μg TG was consumed by 10^9 spermatozoa of *A. lixula* during incubation for 1 h at 20°C (Fig. 2a). The amount of O_2 required to oxidize the fatty acid from this TG was calculated by basically the same procedure used to calculate PC consumption in *P. lividus* spermatozoa. The theoretical value is about 0.25 μmol O_2 in 10^9 spermatozoa, which correlates well with the actual consumption (Fig. 3).

In mammalian species, carbohydrate in the seminal plasma and female reproductive tract has been postulated to be responsible for the motility of spermatozoa (Peterson and Freund, 1976). Without seminal plasma, however, mammalian spermatozoa can maintain motility under aerobic conditions (Lardy and Phillips, 1941a). During incubation, the amount of endogenous phospholipid diminishes (Lardy and Phillips, 1941b). Thus, mammalian spermatozoa may also be capable of using endogenous lipids, particularly phospholipids, for energy metabolism.

It is interesting that the metabolic system responsible for energy production in the spermatozoa of sea urchins differs in the orders Arbacioida and Echinoida. On morphological grounds, it is generally considered that sea urchins of the order Echinoida have diverged further than those of the order Arbacioida (Mortensen, 1943; Durham and Melville, 1957; Shigei, 1974). Accordingly, replacement of TG by PC as the substrate may reflect differentiation or specialization of spermatozoa in the Echinoida. Presumably sea urchins of the order Echinoida, which have lost TG as a source of energy in their spermatozoa, are provided with a metabolic system that uses phospholipids, particularly PC.

Acknowledgments

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